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ISOLATION AND CHARACTERIZATION OF ACTIVE PRINCIPLES FROM *RHYNCHOSTYLIS RETUSA* ROOT EXTRACT

SYNOPSIS

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Submitted by

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Introduction:

God gives his blessings to human beings and animals in the form of valuable plants on the earth. Every part of plant whether it is a root, leaf, flower, stem or fruit overcomes the basic needs of man and animals. A natural product is a pharmacologically or biologically active chemical compound(s) or substance(s) produced by living organisms. Natural products may include alkaloids, flavonoids, steroids, sugars, terpenoids etc. as well as pure compounds which are isolated from plant or microorganisms or different parts like leaves, flowers or roots. Natural products are of two types i.e. primary metabolites and secondary metabolites. The metabolites which are required for the growth and maintenance of cellular function are called primary metabolites. These consist of vitamins, amino acids, organic acids, lipids, nucleosides, glycogen etc. These are produced in large quantities and their extraction is simple. On the other hand secondary metabolites are defined as heterogeneous low molecular weight compounds that are not directly required for organism's growth and are produced after the completion of growth phase of organisms. These include antibiotics, alkaloids, toxins, fatty acids, ketones etc. These are present in very low quantities and their extraction is quite difficult (Sarker& Nahar, 2012; Dias et al., 2012).

According to Shafaei et al., (2011) herbal drugs are used for the treatment of various diseases over 40,000 years, comes from the natural products which are derived from plants. Estimated 35,000 plants species out of 2,50,000 to 3,50,000 are used as herbal medicine by 80 percent of peoples of rural areas all over the world (WHO). Drugs taxol/paclitaxel, Vinblastine, Topotecan, Etoposide, Teniposide etc. are derived from medicinal plants having both pharmacological and phytochemical properties. The various research organizations and 200 companies of the world tried to identify plant and animal compounds having medicinal values in the mid-1990s (Kong et al., 2003). In India, there is variety of plants due to different climatic conditions. About 17,500 flowering plant species are known in which 10,000 are herbs (Rout, 2004). The ancient Greeks first time noticed these strange plants and Discorides (1st century A.D) adopted the name orchid in "*Materia Medica*" for these plants. "*Vanda*" name is also mentioned in the Indian Vedic scriptures for these orchids and also it is the generic name of one of the existing orchids (Chugh et al., 2009). Except arctic, hot deserts, aquatic and marine eco systems, orchids are found all over the world (Behera et al., 2013). Orchids are the wonderful gift given by the nature. Orchids are the largest and diverse group of angiosperms, belongs to

second largest family orchidaceae, having 25,000 species with more than 850 genera like *Vanilla, Gastrodia, Bletilla, Dendrobium, luisia, Calanthe, Coelogyms, Cymbidium, Inter alia* etc. India is native to orchids having 2500 species with 167 genera. (Gutierrez, 2010; Singh et al., 2012; da Silva, 2013). Orchid is an epiphyte and herbaceous plant (Chugh et al., 2009). Reinikka, (1995) mentioned that in Ayurveda, Siddha Yunani, Homeopathy, Traditional Chinese medicine (TCM) systems, orchids are used as a traditional system of medicine. Fallarino, (1994) around 1000BC, Susruta and Charaka documented the Ayurveda system. According to Kong et al., (2003), Hindu sacared*Rig-veda* is the bundle of herbal medicines which is the base of health care called Ayurvedic medicine. *Sankeroot*and *Rauwolfia Serpentine* are the wonderful plants of Rig-veda and used as sedative years back ago. Nowadays for the treatment of high blood pressure, their active components are used in western medicines. Kutumbiah, (1962) described that in "*Ayurveda*", medicinal plants are mentioned along with their uses. "Chark" (125 A.D), 1st time introduced Indian orchids to the world and described "*Vanda*" and other orchids like *Flickingeria, Malaxis and Eulophia* species in his book "*charaksamhita*".

During 19th century the purification of active extracts from medicinal plants were started (Kong et al., 2003). In 1817, German scientist, Friedrich Serturner isolated Morphine (analgesic), an alkaloid as first active principle from plant *opium poppy (papaversomniferum)* (Schmitz, 1985). In 1820, the bark of cinchona tree was used to isolate active principle, Quinine (antimalarial). Natural product *"salicin"* from plant *'willow'* is isolated in 1838 by Johannes Buchner. 1st time ancient Greeks used the bark of this plant as a pain reliever. Bayer Company formed its derivative acetyl- salicylic acid or aspirin. This became the significant approach towards the isolation of natural products from plants. So, 1800's was the golden era for natural products (Woodward and Doering, 1944; Yun et al., 2012). Medicinal plants are used as folk medicinal remedies by indigenous peoples throughout the world. Kavirajes are the folk medicinal practitioners in Bangladesh and primary health care is given by the kavirajes in rural areas. They used medicinal plants for the treatment of diabetes, hypertension, rheumatism and heart disorder (Das et al., 2012).

In Himachal Pradesh, India, there are 85 species of orchids under 44 genera (Vij et al., 2013) which has ornamental (orchids like*Aeridesmulliflora, calanthe sp, Rhynchostylisretusa, vandacristata,* etc) and therapeutic (orchids like*Dactylorhizahatagirea, Eulophiadabia, vandatestacea,* etc.) uses (Vij et al., 2011). The phytochemicals like alkaloids, glycosides, flavonoids, bibenzyl derivatives,

phenanthrecenes in orchids are main reason of their therapeutic use (Hossain, 2011) and active against pathogens like fungi, virus, algae and bacteria as antimicrobial or antiviral agents (Singh et al., 2012). Also many phytochemicals are identified from orchids such as 2,6-Dimethoxy–1,4–benzoquinone, Calanthoside, Scopoletin and scoparone, cymbidine isolated from *cymbidium sp, calanthediscolor* and *calanthe liukivensis, dendrobium densiflorum* and *cymbidium goeringii*respectively (Hausen et al., 1984; Yoshikawa et al., 1998; Fan et al., 2001; Singh et al., 2012). The medicinal properties of orchids depend upon their active principles which are yet to be identified with accuracy (Kant et al., 2012). According to Newman and Cragg, (2007) from 1981 to 2006, the natural products and their derivatives have played a significant role in the field of drug discovery and development. About 42 percent of anticancerous drugs and 28 percent of other chemical entities produced from orchid species. From the orchidaceae plants, the various constituents of secondary metabolites are reported (Kong et al., 2003). Today 25 percent of drugs derived from secondary metabolites acts as analgesics (morphine), antiparasitics (quinine), anticholinergics (atropine) and antineoplastics (vinblastine) etc. (Yun et al., 2012). The phytochemical and pharmacological properties of orchids are studied as traditional medicines by different ethnic groups of Nepal and its neighboring countries (Chand et al., 2016). *Luisiatrichorhiza* leaves used to cure chronic wounds and pseudobulbs of *Otochilusalbus* used for bone fractures. *Rhynchostylis retusa* leaves are used to cure rheumatism, *vandacristata* leaves powder is used as a expectorant and also its paste is used to cure cuts and wounds (Das et al., 2012; Subedi et al., 2013). Orchids have significant role in floriculture and medicinal industry (Bhattacharjee et al., 2015) and are highly valued in national and international markets (Kumar et al., 2002). Orchids are used as herbal drugs/nutraceuticals, phytochemicals, cosmetics, fragrant and edible purposes. Due to wonderful horticultural and medicinal significance, orchidaceae family attracts the whole world to study their taxonomy, cytology, chemistry and cultivation etc. (Singh et al, 2016). According to Theophrastus (370-287 BC) the characteristics of these medicinal plants/herbs are changed through their cultivation. Also the orchid industry is promoted by the breeding, *in-vitro* and *in-vivo*microprogation of these orchid species (Hegde, 1997). The conservation and propagation of orchids are carried out through micropropagation of cell, tissue and organ culture (da silva, 2013).

The genus *Rhynchostylis* belongs to *orchidaceae* family under the tribe*Vandeae* and sub tribe *Aeridine* (Dressler, 1993) having 4 species *Rhynchostylis gigantea (R. gigantea), Rhynchostylis retusa (R .retusa), Rhynchostylis coelestis (R. coelestis)* and *Rhynchostylis*

rieferii (*R. rieferii*) which are endemic in Myanmar, Thailand and Southeast Asian countries (Anuttato et al., 2017). *Rhynchostylis cymifera*, one more new species of genus *Rhynchostylis* was identified from Nilambar forests, Malappuram district, Kerela (yohannam et al., 2018). Among these *R. retusa*, an Indian ornamental orchid is a monopodial epiphyte (vij et al., 1984). It is also known as fox tail orchid because it has long tapering, densely packed inflorescences having sweet fragrance. In Myanmar it is named as "Queen of orchids" by flower lovers (Naing et al., 2010). *R. retusa* is known by various names in different languages of India like Kopouphul /kepou in Assamese, Cat tail or fox tail orchid in English, Panaskoli in Konkani, Gajara or Seetechiveni in Marathi, Draupadi mala in Hindi (sexana, 2020), Bhanguru in local language of Hamirpur district of Himachal Pradesh. According to Bhattacharjee and Islam, (2015), it is an endangered epiphyte growing in moist places during monsoon season. It has no endosperm, so requires endophytic mycorrhizae fungi as a source of carbon (mycotrophy) in a parasitic alliance to stimulate symbiotic seed germination which helps in seedling protraction to become an adult plant (Naing et al., 2010). *R. retusa* is found in Sri Lanka, Bhutan, India, China, Malaysia, Jawa, Indonesia, Nepal, Yunnan, Singapore, Bangladesh, Andaman and Nicobar islands, Indochina, Philippines, Mayanmar, Benin, Cambodia, Laos, Thailand and Vietnam (Bhattacharjee and Islam, 2015). According to Saxsena, (2020), it is native to India and distributed in Assam, Kerala, Manipur, Himachal Pradesh, Nagaland, Sikkim, Tripura, Orissa, Arunachal Pradesh, West Bengal, Maharashtra, Chhatisgarh, Uttarakhand, Karnataka, and Madhya Pradesh. According to Adhikari and Fischer, (2011), *R.retusa* is not host specific orchid species as it is found on different host trees like *Alnusnepalensis, Ficusreligiosa, Schimawallichii, Mangiferaindica, Zizyphus* etc. According to Arditti and Pridgeon, (2013), it is the state flower of Assam and also considered as a symbol of love, fertility and merriment. In Malabar district it is used to treat asthma, tuberculosis, nervous twitching, cramp, vertigo, kidney stone, menstrual disorders and palpitations. In India and Nepal, it is used as an emollient and used to cure rheumatism throughout the Indian subcontinent. It is used to cure paralysis by the folk medicinal practitioners (FMP) of Naogaon district, its leaves are used to cure rheumatic pain in Kurigram district, Bangladesh (Rohani et al., 2018; Das et al., 2012). The leaf juice and roots are used as a folk medicine for ear pain and cleaning (Basumatary et al., 2004), juice of roots to cure malarial fever, cut and wounds (Tiwari et al., 2012) and it also shows antibacterial activity against *Bacillus subtilis* and *Escherichia coli* (Hussain, 2011). Due to

environmental degradation and loss of biodiversity the human beings are captured by many adverse diseases like cancer, AIDS, SARS, bird flu etc. so, there is a need of isolation of new and advantageous compounds which provide aid and relief in all facet of the human conditions (Guo et al., 2008).

Literature Review:

The natural products i.e. secondary metabolites have been used as traditional medicines to cure various ailments from thousands years ago. Before 3000 BC (Ayurveda, knowledge of life), Chinese traditional medicine introduced the plants having medicinal properties and their natural products. Dating from 1550 BC the best Egyptian pharmaceutical record "*Ebers Papyrus*" contained 700 crude drugs derived from natural sources. The first documented record of natural products exists in Chinese Materia Medica dating from about 1100 BC, which contained more than 600 medicinal plants. Hippocrates "*The Father of Medicine*" (460-377BC) and Theophrastus (370-287BC) described the medicinal properties of various plants also Theophrastus observed that the characteristics of medicinal plants/herbs were changed through their cultivation. Galen (131-200AD), practiced botanical medicines and made them popular in the West (Fallarino, 1994; Cragg and Newman, 2005). During 19th century the purification of active extracts from medicinal plants were started (Kong et al., 2003). In 1817, German scientist, Friedrich Serturner isolated Morphine (analgesic), an alkaloid as first active principle from plant *opium poppy (papaversomniferum)* (schmitz, 1985). In 1820, the bark of *cinchona* tree was used to isolate active principle, Quinine (woodward and Doering, 1944). (Farnsworth et al., 1985; Smet., 1997; Cragg and Newman, 1997), mentioned that from 1959 to 1980, about 25% active principles were derived from higher plants. Out of 119 chemical substances (United state) from 90 plants, 74% were isolated as a active principles from plants which were used in traditional medicine. Also Food and Drug Administration, United States, 1983 to 1992, found that 157 of 520 drugs (30%) were natural products and their derivatives. Newman and Cragg, (2007), also mentioned that from 1981 to 2006 about 42 percent of anti cancerous drugs and 28 percent of other chemical entities produced from plants and from 2014 to 2016 (Newman and Cragg, 2020), FDA approved 12 drugs which were derived from natural products. According to Kong et al., (2003); Hossain, (2011); Singh et al., (2012), estimated 35000 plant species out of 2,50,000 to 3,50,000 are used as herbal medicine by 80% of peoples of rural areas all over the world because of their phytochemicals like alkaloids, glycosides, flavonoids

etc. which are active against pathogens as antimicrobial, antiviral, anti-inflammatory agents etc.

Churchill et al., (1973) highlighted the different growth patterns of callus culture of leaf tips of *Epidendrum* seedling in H, K and M media and calli was developed only on MS media, also leaf tips of mature *Laeliocattleya* cultured into plantlets only in H and M media. Because both the species have different genera so have different requirements for culture. Vij et al., (1984) observed the leaf segment culture for the propagation of *R. retusa Bl.* through direct organogenesis on BM medium along with auxins (IAA, NAA), Kinetin (KN) and supplements like peptone, urea and casein hydrolysate. Hegde, (1997) introduced the world to Indian orchids stating that India has 1,100 species of orchids with 163 genera comparable to worlds 17,000 species with 750 genera. About 150 species with genera *Aerides, Rhyncostylis, Vanda, Dendrodium*, etc. are ornamental with commercial value and about 25 species have medicinal values. This paper also highlighted that orchid industry was promoted by the breeding and propagation *in vitro* and *in vivo* by introducing exotic species and hybrids of *Vanda, Cymbidium, Cattleya, Dendrobium* etc. and also traces the history of orchidology from Roxburg's time since 1832. Fan et al., (2001) isolated various chemical constituents like 5-[2'-(3"-hydroxy-5"-methoxyphenyl)-ethyl]-1,3-benzodioxole and 7-hydroxy-2-methoxy-1,4-phenanthrenedione along with 16 compounds from the stem extract of *Dendrobium densiflorum* with column chromatography, IR, UV and NMR spectroscopy. Kumar et al., (2002) focused on the *in-vitro* propagation of two orchids *R. retusa (L.)Bl.* and *Cymbidium elegans Lindl.* which was carried by using Green pod culture technique on Murashige and skoog (1962;MS) medium. The seed generation was increased with BAP or IAA into the BM containing casein hydrolysate medium. Phytagel and PAR light also promoted the multiplication and *in vitro* growth. The rate of seed germination was high in *R. retusa* in response to MS medium containing sucrose as compare to *Cymbidium elegans*.

Kong et al., (2003) elaborates the phytochemical and pharmacological properties of various Orchids like *BletillaSriata, Epidendrum Linnaeus, Eriopsislindley, Oncidium Swartz* etc. which are used as herbal medicines. Food and drug administration (FDA) approved seven plants derived anticanceous drug for their commercial uses e.g. taxel/paclitaxel chemical was derived from *Taxus breuifolia* and used for the treatment of tumorous cancer and breast cancer. Vinblastine and vincristine were derived from *Madagascar periwinkle* and used as an antileukemic drug, also Teniposide, Etoposide,

Irinotecan and Topotecan were also derived from plants. Ho and Chen, (2003) discussed the cytotoxic effect of Moscatilin, an anticancerous agent, isolated from the stem of *Dendrobium Loddigesii* orchid, used against the cancerous cell and its active potential for chemotherapy was studied.

Cragg and Newman, (2005) focused on Biodiversity: A continuous source of novel drugs for the millennia and described the current status of drug discovery, combinatorial chemistry and natural products and also the essential factors in drug discovery and development. Attri et al., (2007) studied the post pollination biochemical changes in the floral organs of *R. retusa (L) Bl.* and *Aerides multiflora Robx* and it was observed that both differ in various growth parameters of their life span i.e. senescence after pollination occur in 24 days in *R.retusa* compared to 17 days in *A. multiflora* similarly senescence in pollinated flower occur in 7DAP (days after pollination), 5DAP respectively. The pollinated flowers *of A. multiflora* were highest in sugar and enzyme activity especially in floral lips and perianths as compared to *R. retusa*. Thomas and Michael, (2007), germinated the seeds of *R. retusa* in vitro on ½ MS medium with different concentrations of coconut milk. After incubation of 90 days maximum of 93% cultures produced seedling length of 0.5cm individual seedling were again sub cultured on MS medium having various concentration of BA and NAA, with or without charcoal (AC) and maximum seedling growth of 2.3cm was observed after 1 month on MS medium along with 6 micro molar (µm) NAA and 1.0 g/l of AC. also seedling were sub cultured on MS medium with 2 micro molar(um) TDZ in the presence of AC,8.2 maximum shoot no was observed and shoots were rooted on ½ MS medium having 2 micro molar (µm), IBA and transplanted to soil. Guo et al., (2008) focused on the bioactive natural products which were isolated from endophytes and their importance in medicine, agriculture and industry. Parab and Krishnan, (2008) studied the PCR based RAPD and ISSR markers which were used to find out the genetic variations among the different population of *R. retusa*. 74, RAPD and 30, ISSR fragments were studied from 13, RAPD and 7, ISSR primers respectively. RAPD (76.13%) showed high level of polymorphism than ISSR (62.6%). In RAPD Nei's average genetic identities value was 0.405 to 0.932 and for ISSR, it was 0.733 to 0.933 for different populations of *R. retusa*. Chugh et al., (2009) focused on the potential of different explants in the micropropagation of orchids by using different vegetative parts of a plant instead of using seeds.

Naing et al., (2010) studied the micropropagation of wild medicinal orchid *Coelogynecristata* which was done through culture of shoot axis, intact seedlings and

pseudobulbs segments with different media. It was observed that the culture of seedlings on MS media with combination of 1.0mgL^{-1} NAA and 1.0mgL^{-1} BA without AC (activated charcoal) have shown best results and is best for *in-vitro* propagation of this endemic wild orchid. They further studied the micropropagation of orchid *R. retusa* which was carried out by culturing of root explants, leaf explants and seedling of *R. retusa* on MS, 1/2MS, HP and VW medium. It was observed that seedling shows maximum growth on 1/2MS medium but no growth shown by leaf explants on same medium. It was also observed that activated charcoal should be used for *in-vitro* propagation to reduce exudation of phenolic compounds.

Adhikari and Fischer, (2011) highlights the influence of human induced environmental factors on the recent distribution pattern of *R. retusa* which are already influenced by natural microhabitat conditions like bark pH, bark roughness, tree age and size, light intensity and bark water holding capacity. Singh et al., (2012) reviewed the different orchids like *Dendrobium densiflorum, Calanthe discolor, Cymbidium* etc. along with their phytochemicals and medicinal properties. It was observed that 214 species belonging to 64 genera contained various active components like alkaloids, flavonoids, terpenoids, phenanthrenes and many biologically active compounds having antimicrobial, anti-inflammatory, antitumor ,antioxidant and other medicinal properties. Kant et al., (2012) described the distribution pattern, survival threats and conservation of our *'Astavarga'* orchids in Himachal Pradesh by adopting *in-situ* and *ex-situ* conservation strategies to save the therapeutically important orchids to accelerate the rate of drug discovery. Yun et al., (2012) reported the isolation and cell culture of Cambial meristematic cells (CMC's) in the sustainable production of natural products by providing controlled conditions to meet the stringent requirements for drug production. Das et al., (2012) described the ethno-medicinal properties of 64 orchids like *R. retusa, Murrayakoenigii, Dautra metal L*, etc. which are used to cure various diseases like anemia, bone fracture, chicken pox ,rheumatism, heart disorder, hypertension etc. by folk medicinal practitioners of Kurigram district, Bangladesh. Amin et al., (2012) studied the analgesic and anti- inflammatory activities in the methanolic leaf extract of *R. retusa* plant in acetic acid induced writhing and carrageenan, formaldehyde induced paw edema respectively. Both the activities were found to be dose dependent. Doses of 200 mg/kg and 400 mg/kg showed 28.84 % and 35.81 % inhibition of acetic induced writhing and doses of 100 mg/kg, 200 mg/kg and 400 mg/kg showed 7.80 %, 8.67 % and 14.32 % mean inhibition of carrageenan induced paw edema respectively. At doses of 200 mg/kg

and 400 mg/kg, the significant ($p<0.01$, $p<0.001$) anti-inflammatory activity of formaldehyde induced mice paw edema was observed. Behera et al., (2013) highlighted the importance of medicinal orchids of India like etc and their floricultural and traditional medicinal values, also *in vitro* conservation of rare or endangered orchids was carried out through micropropagation. da Silva, (2013) studied the importance of cell, tissue and organ culture, micropropagation and genetics or transformations of orchids and also discussed how these biotechnological techniques has helped in the improvement and conservation of orchids specially of pharmacological interest. It also helps to explore the various phytochemicals and secondary metabolites from medically important orchids.

Radhika et al., (2013) highlighted the antifungal activity and cytotoxic activities of *R. retusa* in chloroform, methanol and hexane extract. Antifungal activity was estimated by well diffusion method at different concentration (500, 250, 100mg/ml) .chloroform extract showed the maximum zone of inhibition (7-14mm) at conc. of 500mg/ml at 30°C after 72hrs. Maximum toxicity was observed at conc. of 1000µg/ml, least at conc. 10µg/ml and brime shrimp lethality (LC_{50}) were found to be 86.41µg/ml, 30.50µg/ml and 18.52µg/ml in Hexane,Choloroform and Methanol extract respectively. Nongdam, (2014) discussed the ethnomedicinal characterstics of 30 medicinal orchids of Nagaland including *R. retusa* and focused on the medicinal and therapeutic applications of these orchids.

Bhattacharjee and Islam, (2015) observed that the seed germination and growth ability of PLB's of *R. retusa* orchid on different media like MS, 1/2MS, BS and PM medium in combination with different plant regulators like BAP, KIN, NAA, and IAA were used in single or in combination. MS medium was found to be thebest for seed germination (Maximum percentage 70-60%) and PLB's formation. This technique and protocol will be used for further research of biomedical compounds isolation, genetic transformation and conservation of endangered orchids. Bhattacharjee and Islam, (2015) studied the antimicrobial activities of different extracts (Methanol, Ethanol, chloroform and Hexane) of *R. retusa* on five different bacteria (gram positive: *Staphytococcus aureus* and *Bacillus Subtilis*; gram negative: *Vibrio Cholerae*, *Escherichia coli* and *Klebsiella pneumonia*) and three fungal species (*Penicillium sp.*, *Rhizopus sp.* and *Aspergillus niger*) using Disc diffusion method. The inhibition zone ranged from 5-12mM in antibacterial activity for all bacteria. Chloroform extract with the conc.10.0mg/ml showed the highest inhibition zone (10-12mM) and all the three fungal species showed the highest zone ofinhibition (12-15mM) for antifungal activity. Chand et al., (2016) evaluated the polyphenolic ,

flavonoids and antioxidant activity of nine species of wild orchids of Nepal including *R. retusa* by Folin-ciocalteu's phenol reagent, aluminum chloride and 2,2-Diphenyl-1-picrylhydrazyl free radical scavenging assay respectively. It was concluded that in *R. retusa* leaves, flavonoids content was higher than the content found in the roots of *Gastrochilus acutifoluis (G. acutifoluis),* polyphenolic content and antioxidant activity were found higher in the stem of *Vanda Cristata* than the *G. acutifoluis*. So, the orchids with high level of antioxidant activity can be used for the extraction of natural food additives and drugs. Minh et al., (2016) highlighted the use of ethanol extracts from roots and leaves of 6 hybrid *Phalaenopsis spp.* to find the total phenolics, flavonoids and antioxidant activity of orchid. It was found that antioxidant activity of root extract was higher because of high content of Ferulic, P-coumaric and Sinapic acids as compare to leaf extract. Around eleven phenolic compounds were identified.

Bhatti et al., (2016) propagated *R. retusa* through symbiotic in vitro seed germination. In this study sterilized root segments were taken on oat meal agar medium and three fungal endophytes, (RR201-RR203) observed depending upon the colony and micromorphogical characterstics. The mature seed were co-cultured with these fungal isolates in which initial swelling was observed in embroys after 2.10±0.02 weeks. 75% seeds were ruptured after 4.62± 0.18 weeks irrespective of the fungal isolates but only isolate RR202 showed the morphogenetic changes and after 12.50±0.50 weeks, protocorns were developed also after 18.00±0.71 and 26.80±0.57 weeks the leaf and roots were emerged. The survival rate of seedlings 98.34±0.28% was observed. Anuttato et al., (2017) discussed the morphological charactersticsof *R. gigantea* in Thailand by clustering analysis through an UPGMA approach (Clustering analysis using Unweighted Pair Group Method with Arithmatic Mean) by taking *R. retusa* and *R. colestics* as the outgroup samples. Sembi et al., (2017) studied the effect of ascorbic acid at different concentrations (5, 10, 15and 20 mM) on the growth and survival of encapsulated PLB's (protocorn like bodies) of *R. retusa*when kept at 25°C. 90% germination potential of PLB's with 15mM ascorbic acid was observed after 32 weeks. At low conc. of ascorbic acid (5mM) better differentiation of PLB's into plantlets was observed as compared to higher levels (15,20mM),Also at higher level the multiplication of secondary PLB's was found to be high. Bhatnagar et al., (2017) presented first report on antimycobacterial and leishmanicidal activities of *R. retusa, Ttropidiacurculiodides and Satyriumnepalense,* orchids of Arunachal Pradesh, India. MIC of 15.7 µg/ml of the n-hexane fraction of the flower of *Satyriumnepalense* showed the significant antimycobacterial activity. The

leishmanicidal activity of the diethyl fraction of the roots of *R. retusa* was observed with IC_{50} value 56.04 and 18.4 µg/ml against promastigotes and intracellular amastigotes respectively.

Devi and Neelashree, (2018) developed a methodology for *in vitro* mass propagation of different explants such as shoot ,leaf and roots of *R. retusa* on different media. Shoot tip explants showed maximum survival and differentitation rate w.r.t .leaf and root tip explants in VW medium supplemented with NAA (0.1 mg/l) along with either Kinetin (1.0mg/l) or BAP (1.0mg/l).with the reduction of Kinetin and BAP and removal of NAA, the growth and development of PLB's (proto corn like bodies) into plantlets of size 3.0-3.5cm were achieved in VW medium.

Rohani et al., (2018) focused on the potential of *R. retusa*, a medicinal orchid to cure paralysis attack by applying the paste of fresh leaves of *R. retusa*, roots of *Datura metal L. (solanaceace)* and twelve to thirteen fruits of *pipernigrum L. (piperaceace)* by folk medicinal practitioner (FMP's) of Nagaon district, Bangladesh. This orchid is also used to cure rheumatism, piles, menstrual disorder, fever, allergy, fractures, blood dysentery, cough and cold and inflammation. Calixto, (2019) mentioned that Brazilian biodiversity being the largest biodiversity in the world, having more than 50,000 species of plants and described the role of natural products in modern drug discovery by highlighting that about 35 percent of medicines are derived from natural products. Deepthi and Ray, (2020) demonstrated the diversity of algal flora in the velaman roots of *R. retusa, Epidendrum radicans, Acampepraemorsa and Oncidiumsphacelatum*and analysed the Nitrogen Fixing Acivity (NFA) of cyanobacteria in the velaman roots of the orchids which will be helpful in the organic cultivation of orchids. Saxena, (2020) documented the research status of *R. retusa* by highlighting its traditional and remedial pharmacological uses and suggested conservational strategies to save this endangered plant.

Research Gap Identification:

There are reports on ethnomedicinal and ethno veterinary properties of *R. retusa.* Researchers have studied the antifungal, cytotoxic, antibacterial , antioxidant ,anti-inflammatory, anti-microbial, antimycobacterial, leishmanicidal and analgesic activities and also discussed the post pollination biochemical changes in floral organs, *in vitro* and *in vivo* micropropagation by using different explants, seed germination and growth ability of PLBs, and cynobacterial nitrogen fixation in the velamen roots of the *R.*

retusa. Inspite of this work it seems that *R. retusa* has not been completely utilized by researchers for their medicinal applications for drug development which depends upon their active principles. Despite of the various researches carried out on orchids no systematic study has been carried on isolation and characterization of active principle from root extract of *R. retusa*. Therefore to cover this gap root extract of *R. retusa* has been taken.

Proposed Objectives: The present work has been chosen with following objectives:

1. Phytochemical analysis and chemical profiling of *R. retusa* roots.
2. To isolate and characterize the active principles of *R. retusa* roots.
3. To assess the biological activity of pure isolated compound.
4. Regional distribution of constituents in *R. retusa* roots.

Proposed Methodology: The following steps will be taken during the research work:

1. Sampling of plant material: The roots of species *R. retusa* (family-Orchidaceae) will be collected from the trunks and branches of old Mango trees during Feb to March, 2020 from various regions of the Hamirpur, Bilaspur, Mandi and Kangra district of Himachal Pradesh, India.

Figure 1: *R. retusa* plant.

The roots will be separated from plants, thoroughly washed under tap water to make them free from foreign substances and then dried in dark at room temperature. The dried roots will be powdered with electric blender and stored in air tight container for further analysis.

2. Phytochemical analysis and chemical profiling of the powered roots of *R. retusa*: The ash value, moisture content, extractive value and pH of the powdered material shall be studied. In addition to the above studies, the total phenolic, total flavonoid and total polyphenol content of the plant will be determined from plant extract. (Partap et al.,2019; Shukla et al.,2012).

3. Preparation of sample extract: The dried powdered root material will be dissolved in organic solvents like methanol, ethanol chloroform etc. and followed by a period of percolation. Then liquid extract will be dried and dried solid extract will be kept for further investigation. (Sasidharan et al., 2011; Sharma and Singh, 2013).

4. Isolation of active principles: The isolation of bioactive compounds from the root extract of *R. retusa* will be done by different separation techniques like TLC, LCMS, Column chromatography and High Performance Liquid Chromatography (HPLC) (Sasidharan et al., 2011).

5. Characterisation and Structural elucidation of pure active principles: This will be done by the use of spectroscopic techniques like UV, IR, NMR etc. (Sasidharan et al., 2011).

6. Biological Activity: The biological activity is to be determined by standard protocol (Amin et al., 2011; Radhika et al., 2013; Bhattacharjee and Islam, 2015).

7. Regional distribution of constituents in *R. retusa* roots: It will be done by determining the total phenolic, total flavonoid, total polyphenol content and biological activity of the roots of plants obtained from various regions of Himachal Pradesh in order to assess the variation in the parameters mentioned above with respect to climatic and geographical variations in the growing conditions of the orchids (Partap et al., 2019; Baba and Malik, 2015; World Health Organization, 2011).

Thesis Outline: Thesis will be comprised of five chapters as:

Chapter 1: *Introduction:-* Includes introduction of natural products, phytochemicals, orchidaceae family along with genera and species as well as applications of orchids. The

various active principles derived from different plants and their ethno-medicinal and ethno-veterinary properties.

Chapter 2: *Literature Review:-* Includes uses of natural products before 3000BC to till date. Churchill et al., 1973 to Saxena, 2020 covered different methods of propagation of endangered orchids and their antimicrobial properties, phytochemicals and their medicinal properties as well as active components of various orchids.

Chapter 3: *Methodology:* - Includes the methods which will be used for the extraction and isolation of the active principles from *R. retusa root* extract and also the characterization and structural elucidation of pure active principles with the help of various spectroscopic techniques like UV, IR, NMR etc. It also covers the assessment of the biological activities of the isolated compounds along with the total flavonoid, total polyphenol and total phenolic content of plant root extract.

Chapter 4: *Results and Discussion:-* The results shall be elaborated and discussed for future scope.

Chapter 5:*Summary andConclusion:* - Finally conclusion will be drawn from the results.

Chapter 6: *References:* - All the references will be written as per Harvard guidelines.

Research Plan Schedule: Research plan under the topic "Isolation and Characterization of Active Principles from *Rhynchostylis retusa* Root Extract" is as under:

Year 1:

- Review of Literature
- Design of work
- Selection of Methodology
- Submission of Synopsis

Year 2:

- Sample collection
- Extraction and Isolation of active principles
- Characterization and Structural elucidation of the compounds
- Chemical Profiling
- Seminar/Conference publications/presentations

Year 3:

- Results and conclusion
- Seminar/Conference publications/presentations

- Research Publications
- Writing and Submission of Thesis

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CERTIFICATE
(SYNOPSIS APPROVAL CERTIFICATE)

This is to certify that the Revised Synopsis for Ph.D. Thesis titled, **"Isolation and Characterization of Active Principles from *Rhynchostylis retusa* Root Extract"**, submitted by **Ms. Anjna Kumari** to the Career Point University, Hamirpur (H.P.), India in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in theDepartment of Chemistry, School of Basic and Applied Sciences has been approved by the Research Advisory Committee (RAC) after an Oral Examination of the student on dated 28/07/2021.

09/08/2021

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